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14. ABSTRACT The hypothesis being tested in this proposal is that breast cancer cells, especially metastatic cells, are prone to rapid increases in ROS and lipid peroxidation caused by adverse micro-environmental conditions and that these oxidative bursts could have deleterious consequences including ferroptosis. Ferroptosis is defined as an iron-dependent, non-apoptotic form of programmed cell death characterized by the accumulation of intracellular soluble and lipid reactive oxygen species (ROS). For this reason, breast tumor cells must acquire mechanisms to protect against oxidative bursts and ferroptosis to metastasize. Work accomplished during the first year of this proposal has demonstrated that the $\alpha 6 \beta 4$ integrin protects breast tumor cells against ferroptosis caused by drugs that induce this form of cell death, as well as by detachment from the extracellular matrix. The work performed also revealed that the mechanism involves the ability of $\alpha 6 \beta 4$ integrin signaling to sustain expression of glutathione peroxidase 4 (GPX4), an enzyme that reduces lipid peroxides and promotes resistance to ferroptosis.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	3
2. Keywords.....	3
3. Accomplishments.....	3
4. Impact.....	4
5. Changes/Problems.....	5
6. Products.....	5
7. Participants & Other Collaborating Organizations.....	5
8. Special Reporting Requirements.....	5
9. Appendices.....	5

1. INTRODUCTION

The goal of this Breakthrough Award application is to open a new direction in our understanding of the mechanisms that sustain the survival of breast tumor cells, a problem of paramount importance in the biology and therapy of breast cancer. Adverse conditions in the tumor microenvironment such as detachment from matrix (anoikis) and hypoxia result in cell death, and tumor cells must acquire mechanisms to resist such death to survive and progress to metastatic disease. Our interest in this area has been awakened by the discovery of a novel mode of programmed cell death termed ferroptosis, which is defined as an iron-dependent, non-apoptotic form of programmed cell death characterized by the accumulation of intracellular soluble and lipid reactive oxygen species (ROS). The overarching hypothesis to be tested is that breast cancer cells, especially metastatic cells, are prone to rapid increases in ROS and lipid peroxidation caused by adverse micro-environmental conditions and that these oxidative bursts could have deleterious consequences including ferroptosis. For this reason, breast tumor cells must acquire mechanisms to protect against oxidative bursts and ferroptosis to metastasize. One novel mechanism involves expression of the $\alpha 6 \beta 4$ integrin, which we postulated in the original application has the ability to regulate enzymes that buffer intracellular ROS and diminish lipid peroxidation.

Two specific aims were proposed to address our hypothesis. The first aim was to establish that the $\alpha 6 \beta 4$ integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments and the second aim was to determine the mechanism by which the $\alpha 6 \beta 4$ integrin protects tumor cells against ferroptosis. As reported below, we have made significant progress in achieving the goals that we outlined and the impact of the data that we have obtained has been strengthened by other developments in the field.

2. KEYWORDS

Breast cancer, Integrins, Ferroptosis, Cell Death, Metastasis, Lipid Peroxidation

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals of the project were divided into two major tasks:

Task 1. Establish that the $\alpha 6 \beta 4$ integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments. Months 1-24.

Task 2. Determine the mechanism by which the $\alpha 6 \beta 4$ integrin protects tumor cells against ferroptosis. Months 6-24.

What was accomplished under these goals?

Task 1. Establish that the $\alpha 6 \beta 4$ integrin protects breast tumor cells against ferroptosis caused by adverse tumor microenvironments. We have accomplished much of this task, which has enabled us to publish our first manuscript on this project (Brown et al., Journal of Cell Biology, 2017, 216:4287-4297).

1a. Assay ferroptosis as a function of $\alpha 6 \beta 4$ expression in breast cancer cell lines. Using the approaches outlined in the SOW, we have established that ferroptosis resistance is mediated by the $\alpha 6 \beta 4$ integrin in breast cancer cell lines (see Figures 1 and 2 in the Brown et al. publication).

1c. Assay ferroptosis in PDX models of breast cancer. To address this task, we sorted a PDX model of triple-negative breast cancer into $\alpha 6 \beta 4^{\text{high}}$ and $\alpha 6 \beta 4^{\text{low}}$ populations by FACS. Subsequently, we assessed each population for sensitivity to ferroptosis using erastin. The results obtained demonstrated that the $\alpha 6 \beta 4^{\text{high}}$ PDX cells are much more resistant to erastin-mediated ferroptosis than the $\alpha 6 \beta 4^{\text{low}}$ low cells (see Figures 3C and 3D in the Brown et al. publication).

1e. Assess the ability of matrix-detachment to induce ferroptosis. Completion of this task has resulted in a significant advancement in our understanding of the pathophysiological processes that trigger ferroptosis. We

discovered that matrix-detachment of mammary epithelial and breast carcinoma cells results in death by ferroptosis unless they express the $\alpha 6\beta 4$ integrin (see Figure 2 in the Brown et al. publication).

Task 2. Determine the mechanism by which the $\alpha 6\beta 4$ integrin protects tumor cells against ferroptosis. Significant progress has been made on Task 2.

2a. Determine that the $\alpha 6\beta 4$ integrin buffers the increase in ROS and lipid peroxidation that accompanies ferroptosis. We demonstrated that the $\alpha 6\beta 4$ integrin promotes resistance to ferroptosis by buffering the increase in lipid peroxidation that is the root cause of this form of cell death. Moreover, we pursued this problem further and discovered that the mechanism involves the ability of $\alpha 6\beta 4$ signaling to sustain expression of glutathione peroxidase 4 (GPX4), which is the only glutathione peroxidase that reduces lipid peroxidation (see Figure 7 in the Brown et al. publication).

2b. Determine whether the ability of $\alpha 6\beta 4$ to regulate ROS and lipid peroxidation is ligand-dependent. We pursued this task as outlined in the SOW. The results we obtained support the conclusion that the ability of $\alpha 6\beta 4$ to buffer ROS and lipid peroxidation and prevent ferroptosis is actually ligand-independent. We are now studying the mechanism that underlies ligand-independent activation of $\alpha 6\beta 4$.

2e. Assess the role of cysteines in promoting the association of $\alpha 6\beta 4$ with PrxII and protecting against lipid peroxidation and ferroptosis. We have initiated this task by generating expression constructs for the $\beta 4$ integrin subunit in which the 5 cysteine residues that we highlighted in the proposal have been mutated. We are now able to proceed with the functional studies described in this task.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

In the final year of the grant, we plan to finish the remaining sub-task in the first major task (1f), which is to assess the role of ferroptosis in breast cancer using mice. As mentioned above, we have initiated this sub-task using cells isolated from PDX models of breast cancer.

We also plan to complete Task 2. Specifically, we will initiate and complete sub-tasks **2c** (Evaluate the role of PrxII in reducing H_2O_2 levels and protecting breast cancer cells from ferroptosis) and **2d** (Determine that the $\alpha 6\beta 4$ integrin associates with PrxII and localizes it at the plasma membrane). We will also continue with and complete **2e** (Assess the role of cysteines in promoting the association of $\alpha 6\beta 4$ with PrxII and protecting against lipid peroxidation and ferroptosis).

4. IMPACT

The findings that we have made during the first year of this award have significant implications for the therapeutic treatment of aggressive cancers. In particular, our discovery that $\alpha 6\beta 4$ promotes ferroptosis resistance by sustaining ferroptosis resistance is very significant based on the recent report in *Nature* that highly aggressive breast tumor cells are prone to lipid peroxidation and that they must sustain GPX4 expression to prevent ferroptosis (Viswanathan et al., *Nature*, 2017, 547:453-457). This finding implies that the mechanisms used by these breast tumor cells to sustain GPX4 are critical for their survival and aggressive behavior, but the nature of these mechanisms is not known. Clearly, our observation that $\alpha 6\beta 4$ sustains GPX4 expression and activity in stress conditions provides one such mechanism. Our data also infer that these cells are dependent on both $\alpha 6\beta 4$ and GPX4 for their survival and aggressive behavior. A major effort is underway to develop

effective GPX4 inhibitors (Hangauer et al. *Nature*, 2017, 551:247-250) and a similar effort to develop inhibitors of $\alpha\beta4$ signaling seems warranted based on our work.

5. CHANGES/PROBLEMS

Nothing to report.

6. PRODUCTS

The major product to report is our journal publication (Brown CW, Amante JJ, Goel HL, Mercurio AM. The $\alpha\beta4$ integrin promotes resistance to ferroptosis. *Journal of Cell Biology*, 2017, 216:4287-4297). This publication is included in the Appendix.

7. PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS

What individuals worked on the project?

Arthur M. Mercurio (No Change)

Caitlin W. Brown (No Change)

John J. Amante (No Change)

Has there been a change in the active support of PI or key personnel?

No change

What other organizations were involved as partners?

None

8. APPENDICES

We include 1 publication in the Appendix.

The $\alpha 6 \beta 4$ integrin promotes resistance to ferroptosis

Caitlin W. Brown, John J. Amante, Hira Lal Goel, and Arthur M. Mercurio

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Increases in lipid peroxidation can cause ferroptosis, a form of cell death triggered by inhibition of glutathione peroxidase 4 (GPX4), which catalyzes the reduction of lipid peroxides and is a target of ferroptosis inducers, such as erastin. The $\alpha 6 \beta 4$ integrin protects adherent epithelial and carcinoma cells from ferroptosis induced by erastin. In addition, extracellular matrix (ECM) detachment is a physiologic trigger of ferroptosis, which is evaded by $\alpha 6 \beta 4$. The mechanism that enables $\alpha 6 \beta 4$ to evade ferroptosis involves its ability to protect changes in membrane lipids that are proferroptotic. Specifically, $\alpha 6 \beta 4$ -mediated activation of Src and STAT3 suppresses expression of ACSL4, an enzyme that enriches membranes with long polyunsaturated fatty acids and is required for ferroptosis. Adherent cells lacking $\alpha 6 \beta 4$ require an inducer, such as erastin, to undergo ferroptosis because they sustain GPX4 expression, despite their increase in ACSL4. In contrast, ECM detachment of cells lacking $\alpha 6 \beta 4$ is sufficient to trigger ferroptosis because GPX4 is suppressed. This causal link between $\alpha 6 \beta 4$ and ferroptosis has implications for cancer biology and therapy.

Introduction

The ability of cells to resist death is a hallmark of tissue homeostasis and disease, especially cancer (Hanahan and Weinberg, 2011). With respect to cancer, resistance to chemotherapy-induced cell death is a problem of paramount importance (Safa, 2016). In addition, adverse conditions in the tumor microenvironment, such as detachments from matrix (anoikis), result in cell death, and tumor cells must acquire mechanisms to resist such death to survive and progress to metastatic disease (Buchheit et al., 2014). Our interest in this area has been awakened by the discovery of a novel mode of programmed cell death, termed ferroptosis. Ferroptosis is defined as an iron-dependent form of programmed cell death, which is characterized by lipid reactive oxygen species (ROS) accumulation that damages the plasma membrane by peroxidation of polyunsaturated fatty acids (Yang et al., 2016; Yang and Stockwell, 2016). At a mechanistic level, ferroptosis is triggered by the loss of activity for the lipid repair enzyme glutathione peroxidase 4 (GPX4), which catalyzes the reduction of lipid and other peroxides and is a target of several ferroptosis inducers (Yang et al., 2014). The antiporter system X_C^- , which imports cystine into the cell in exchange for glutamate, also has a critical role in protecting against ferroptosis because cysteine, the monomeric form of cystine, is converted to the antioxidant glutathione, which is a substrate for GPX4 (Yang and Stockwell, 2016). Molecules that inhibit system X_C^- , such as erastin, trigger ferroptosis, and they have proven to be useful for studying this process in detail (Dixon et al., 2012).

At present, the significance of ferroptosis in the context of epithelial and carcinoma biology is still emerging. The findings that ferroptosis inducers can inhibit the growth of tumor xenografts have heightened the cancer relevance of this mode of cell death (Yang et al., 2014; Kim et al., 2016). Although exciting, these findings do not provide insight into the mechanisms used by cells to evade ferroptosis or whether tumor cells encounter conditions that trigger ferroptosis and, consequently, whether they must acquire mechanisms to evade this process. The study that reported that p53-mediated tumor suppression involves ferroptosis (Jiang et al., 2015) provided some indication of the physiological relevance of this process in cancer. Ferroptosis also occurs in p53 mutant cells (Jiang et al., 2015) indicating that mechanisms other than loss of p53 function are involved in promoting resistance to ferroptosis.

Given the existing literature, we were intrigued by the possibility that integrin signaling protects cells from ferroptosis. We were particularly interested in the integrin $\alpha 6 \beta 4$ because several seminal studies have revealed that this integrin can protect epithelial and carcinoma cells from death in adverse conditions (Lipscomb and Mercurio, 2005; Giancotti, 2007), and it has been implicated in metastasis. In this study, we uncovered a key role for $\alpha 6 \beta 4$ in the evasion of ferroptosis, and we pursued the mechanisms involved.

Results

Results

The integrin $\alpha 6 \beta 4$ promotes resistance to erastin-induced ferroptosis

Initially, we assessed the susceptibility of MCF-10A (immortalized breast epithelial cells) and SUM-159 (breast carcinoma

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Abbreviations used: DFO, deferoxamine; DPP, 5,15-diphenylporphyrin; GPX4, glutathione peroxidase 4; LDH, lactate dehydrogenase; MDA, malondialdehyde; PAM, protospacer-adjacent motif; PDX, patient-derived xenograft; polyHEMA, 2-hydroxyethyl methacrylate; qPCR, quantitative PCR; ROS, reactive oxygen species; Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

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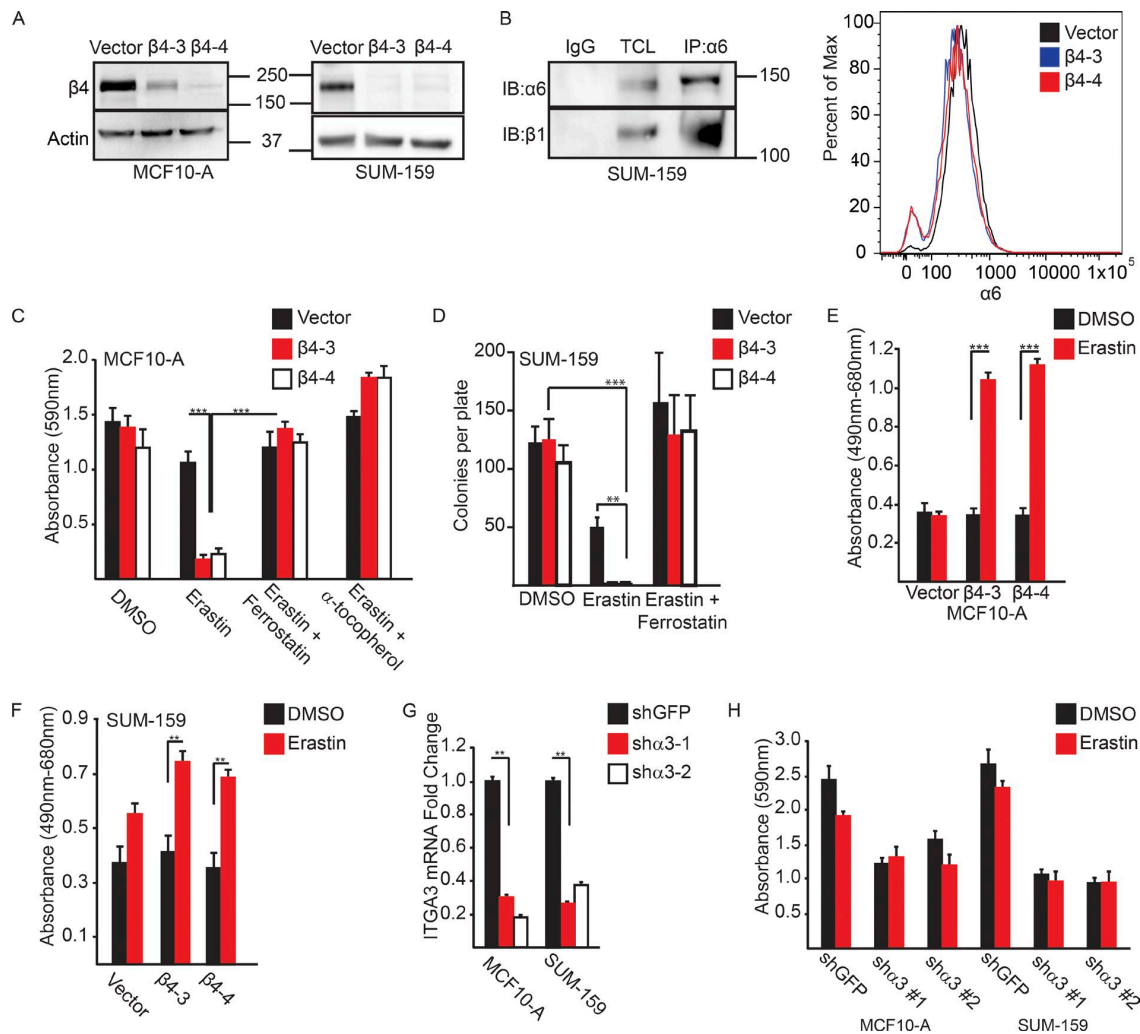


Figure 1. The $\alpha 6 \beta 4$ integrin promotes evasion of ferroptosis induced by erastin. (A) The $\beta 4$ -integrin subunit was depleted in MCF10-A and SUM-159 cells by CRISPR/CAS9 using two independent guide RNAs ($\beta 4$ -3 and $\beta 4$ -4). Depletion of $\beta 4$ expression was verified by immunoblotting. (B) Extracts of $\beta 4$ -depleted cells were immunoprecipitated with an $\alpha 6$ antibody and immunoblotted with a $\beta 1$ antibody to verify that these cells express $\alpha 6 \beta 1$ (left blot). Vector control and $\beta 4$ -depleted cells were assessed for surface expression of the $\alpha 6$ -integrin by flow cytometry. (C and D) Control and $\beta 4$ -depleted MCF10-A or SUM-159 cells (5×10^3) were plated in 60-mm dishes in the presence of either DMSO, 10 μ M erastin, erastin and 2 μ M ferrostatin-1, or erastin and 500 μ M α -tocopherol, and survival was quantified after 7 d by either DMSO extraction of crystal violet-stained cells or colony counting, respectively. (E and F) MCF10-A or SUM-159 vector control and $\beta 4$ -depleted cells were assayed for LDH after 6 h of treatment with either DMSO control or 10 μ M erastin. (G) shRNA-mediated depletion of the $\alpha 3$ -integrin subunit in MCF10-A and SUM-159 cells was confirmed by qPCR after 7 d of puromycin selection. (H) MCF10-A and SUM-159 $\alpha 3$ -depleted cells were plated at clonal density, and survival was quantified as in C and E. All experiments were performed independently three times, and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

cells) to undergo cell death after treatment with erastin, a ferroptosis inducer (Dixon et al., 2012) as a function of $\alpha 6 \beta 4$ expression. For that purpose, we generated a CRISPR/Cas9 deletion of the $\beta 4$ subunit of the $\alpha 6 \beta 4$ heterodimer (Fig. 1 A), leaving the $\alpha 6 \beta 1$ heterodimer intact, as assessed by immunoblotting and flow cytometry (Fig. 1 B). We observed that MCF-10A cells that lacked $\alpha 6 \beta 4$ were significantly less viable in the presence of erastin compared with control cells, as assessed by colony formation assays (Fig. 1 C). The loss of viability in $\alpha 6 \beta 4$ -depleted cells in response to erastin was rescued by the addition of ferrostatin-1, a specific inhibitor of ferroptosis (Dixon et al., 2012), or by the addition of lipophilic antioxidant α -tocopherol (Fig. 1 C). Similar results were obtained with SUM-159 cells (Fig. 1 D). Given that ferroptosis is a form

of programmed necrosis (Dixon et al., 2012), we used the lactate dehydrogenase (LDH) assay to assess cytotoxicity in response to erastin. Treatment of $\alpha 6 \beta 4$ -depleted MCF-10A cells (Fig. 1 E) or SUM-159 cells (Fig. 1 F) with erastin significantly increased extracellular LDH activity, which was not observed with control cells.

An important issue is whether the ability of $\alpha 6 \beta 4$ to evade erastin-induced ferroptosis is specific to this integrin. As shown in Fig. 1 B, $\alpha 6 \beta 4$ -depleted cells expressed the $\alpha 6 \beta 1$ integrin, but that integrin was not sufficient to evade ferroptosis under those conditions. We also targeted the $\alpha 3 \beta 1$ integrin because it is a laminin receptor expressed by epithelial and carcinoma cells (DiPersio et al., 1997). Expression of the $\alpha 3$ subunit was diminished in both MCF-10A and SUM-159 cells

using shRNAs, and $\alpha 3$ mRNA expression was evaluated by quantitative PCR (qPCR; Fig. 1 G). The $\alpha 3$ -depleted cells, in contrast to the $\beta 4$ -depleted cells, were not sensitive to erastin, although they did form significantly fewer colonies than control cells did (Fig. 1 H).

ECM detachment triggers ferroptosis: Evasion by $\alpha 6\beta 4$

The ability of the integrin $\alpha 6\beta 4$ to protect against erastin-induced ferroptosis has important implications for drug targeting. Nonetheless, little is known about pathophysiological stimuli that trigger ferroptosis. Based on previous work indicating that $\alpha 6\beta 4$ can promote cell survival in stress conditions (Bachelder et al., 1999; Zahir et al., 2003), we assessed the susceptibility of $\alpha 6\beta 4$ -depleted cells to ferroptosis after detachment from the ECM. We observed that ECM-detached MCF-10A and SUM-159 cells lacking $\alpha 6\beta 4$, but not control cells, exhibited a substantial decrease in viability after 24 h (Fig. 2 A). Ferrostatin-1 treatment of $\alpha 6\beta 4$ -depleted cells resulted in a partial rescue of the viability of matrix-detached cells (Fig. 2 A). Similar results were obtained with Hs578t cells, another breast cancer cell line (Fig. 2 B). Treatment with other ferroptosis inhibitors, including liproxstatin-1 (Fig. 2 C; Friedmann Angeli et al., 2014), the iron chelator deferoxamine (DFO), trolox, and α -tocopherol (Dixon et al., 2012), also resulted in a partial rescue of cell death caused by matrix detachment and loss of $\alpha 6\beta 4$ (Fig. 2 D). Detachment of $\alpha 6\beta 4$ -depleted cells increased cytotoxicity significantly compared with control cells, as assessed by the LDH assay, and that increase was prevented by ferrostatin-1 (Fig. 2 E).

We also assayed cell viability as a function of time in the presence of ferrostatin-1 using control and $\alpha 6\beta 4$ -depleted cells (Fig. 2 F). That analysis revealed that ferroptosis occurs rapidly in the absence of $\alpha 6\beta 4$ (between 4 and 12 h), which is consistent with the LDH data on adherent cells treated with erastin (Fig. 1, E and F). Because anoikis has been studied primarily as a form of apoptosis (Meredith et al., 1993; Frisch and Francis, 1994), we compared the ability of carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) and ferrostatin-1 to rescue the viability of $\alpha 6\beta 4$ -depleted cells after ECM detachment. Each of those inhibitors individually was able to affect a partial rescue, and the combination of both inhibitors rescued viability completely (Fig. 2 G).

To control for the specificity of $\alpha 6\beta 4$ depletion, we engineered a $\beta 4$ expression construct that could not be targeted by CRISPR-Cas9. Expression of that construct in $\alpha 6\beta 4$ -depleted cells protected those cells from the loss of viability caused by ECM detachment (Fig. 3 A). We also assessed the viability of the MCF10-A and SUM-159 $\alpha 3$ -depleted cells used in Fig. 1 G in detached conditions and found a moderate decrease in viability that was not rescued with ferrostatin-1 (Fig. 3 B).

We extended our analysis of ferroptosis induced by ECM detachment to human breast tumors. For that purpose, we used patient-derived xenografts (PDXs) of triple-negative breast tumors. After isolation, dissociation, and lineage depletion, tumor cells were sorted based on the level of $\beta 4$ surface expression into $\beta 4^{\text{high}}$ and $\beta 4^{\text{low}}$ populations, taking the top and bottom quartiles, respectively (Fig. 3 C). Those two populations were assessed for viability in detached conditions, either in the presence or absence of ferrostatin-1. Under those conditions, the $\beta 4^{\text{low}}$ population was significantly less viable than the $\beta 4^{\text{high}}$ population, and that loss of viability was rescued by ferrostatin-1 (Fig. 3 D).

Src, which is activated by $\alpha 6\beta 4$, protects against ferroptosis

The signaling pathways that enable cells to evade ferroptosis are poorly understood. We focused on the potential role of Src, because several studies have documented the robust activation of Src by $\alpha 6\beta 4$, and examined the functional consequences (Gagnoux-Palacios et al., 2003; Bertotti et al., 2006; Merdek et al., 2007; Dutta and Shaw, 2008; Yang et al., 2010; Sharma et al., 2012; Pavlova et al., 2013; Hoshino et al., 2015). Activation of Src in $\alpha 6\beta 4$ -depleted MCF-10A and SUM-159 cells was significantly reduced compared with vector controls, as assessed by Y418 immunoblotting (Fig. 4 A), indicating that $\alpha 6\beta 4$ promotes Src activation in ECM detachment. To evaluate a causal role for Src activation in ferroptosis evasion, control cells were treated with the Src inhibitor PP2 and assayed for viability 24 h after detachment (Fig. 4 B). Strikingly, Src inhibition decreased cell viability significantly, and that loss of viability was rescued by ferrostatin-1 and other ferroptosis inhibitors (Fig. 4 B).

Next, we assessed Src activation in adherent cells in response to erastin based on the assumption that erastin treatment stresses cells and elicits a response to diminish that stress. Indeed, erastin treatment of control MCF-10A and SUM-159 cells significantly increased Src activation compared with $\alpha 6\beta 4$ -depleted cells (Fig. 4 C). Moreover, the viability of detached, $\alpha 6\beta 4$ -depleted cells was rescued by expression of an inducible, constitutively active Src (Fig. 4 D). These findings support the hypothesis that $\alpha 6\beta 4$ facilitates Src activation in response to stress (ECM detachment or inhibition of the system X_C^- by erastin).

In pursuit of the mechanism by which Src activation protects against ferroptosis, we were intrigued by the recent studies that found the enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4) is required for ferroptosis because it synthesizes polyunsaturated fatty acids that are the primary target of lipid peroxidation (Doll et al., 2017). We quantified *ACSL4* mRNA expression in MCF10-A and SUM-159 control and $\alpha 6\beta 4$ -depleted cells in adherent and detached conditions (Fig. 5 A) and found a significant elevation of *ACSL4* mRNA with the loss of $\alpha 6\beta 4$ in both conditions. This elevation of *ACSL4* expression was also seen at the protein level in $\alpha 6\beta 4$ -depleted cells (Fig. 5 B). Src inhibition increased *ACSL4* expression in control cells (Fig. 5 C), indicating that it has a causal role in regulating this key ferroptotic enzyme. Moreover, reexpression of the $\beta 4$ subunit in $\alpha 6\beta 4$ -depleted cells repressed *ACSL4* (Fig. 5 D), confirming that the repression of *ACSL4* expression is specific to $\alpha 6\beta 4$ -mediated signaling. Inhibition of *ACSL4* using the thiazolidinedione rosiglitazone (Askari et al., 2007; Doll et al., 2017) increased the viability of detached, $\alpha 6\beta 4$ -depleted cells, confirming the key role of *ACSL4* in ferroptosis susceptibility (Fig. 5 E). In addition, expression of constitutively active Src suppressed the expression of *ACSL4* (Fig. 5 F). These in vitro data were substantiated by comparing $\beta 4$ and *ACSL4* expression in a cohort of patients with breast cancer using cBioportal, where a significant inverse correlation was detected (Fig. 5 G).

The preceding data raise the issue of how $\alpha 6\beta 4$ -mediated Src activation represses *ACSL4* expression. We focused on STAT3 because it can be activated by Src (Yu et al., 1995) and, more specifically, by the $\alpha 6\beta 4$ -Src signaling axis (Guo et al., 2006). Moreover, STAT3 can repress, as well as activate, transcription (Niu et al., 2005). Using the ENCODE database, we identified STAT3 binding in several regions of the *ACSL4*

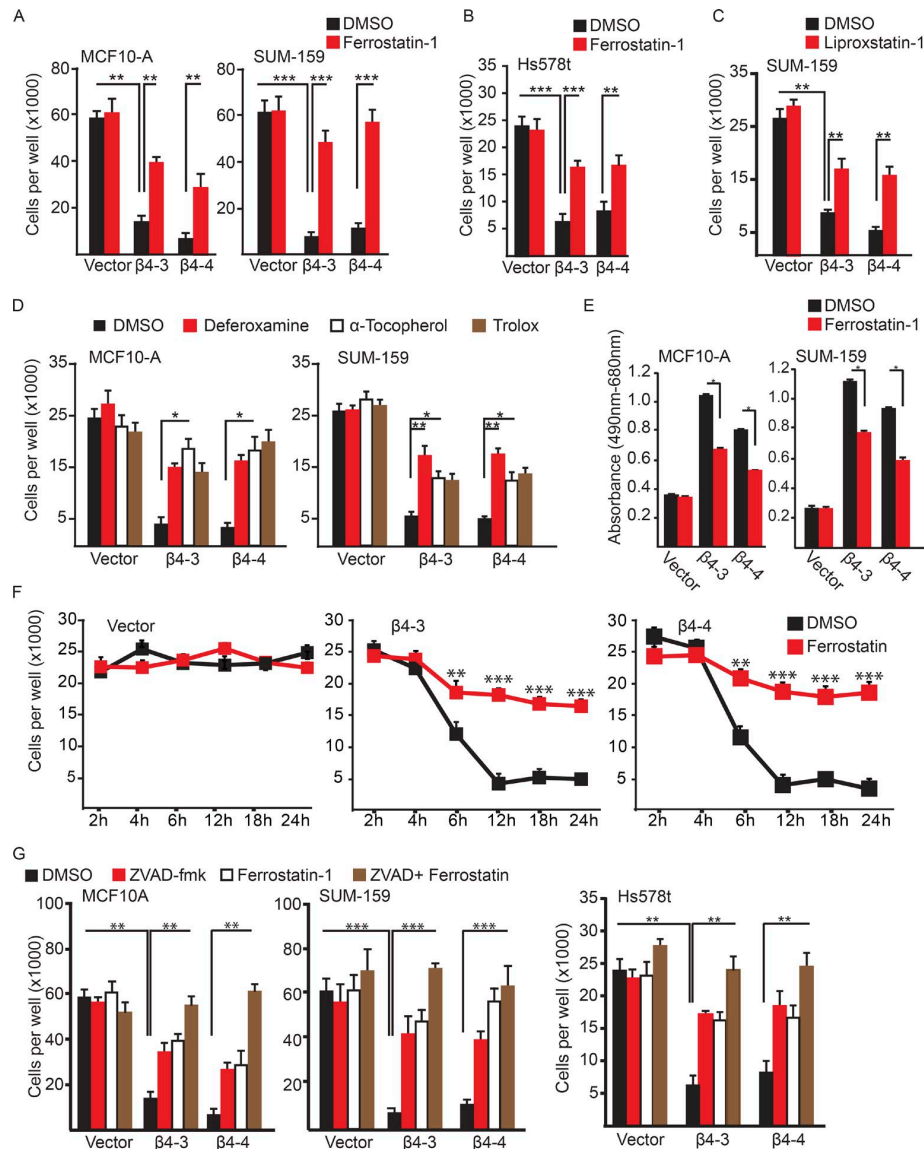


Figure 2. Matrix-deprived cells are susceptible to ferroptosis in the absence of the $\alpha 6 \beta 4$ -integrin. (A) Control and $\beta 4$ -depleted cells were detached for 24 h in the presence of either DMSO or 2 μ M ferrostatin-1, and the number of viable cells was quantified. (B) Control and $\beta 4$ -depleted Hs578t cells were detached for 24 h in the presence of either DMSO or 2 μ M ferrostatin-1, and the number of viable cells was quantified. (C) Control and $\beta 4$ -depleted SUM-159 cells were detached for 24 h in the presence of either DMSO or 2 μ M lipoxystatin-1, and the number of viable cells was quantified. (D) Control and $\beta 4$ -depleted MCF-10A and SUM-159 cells were detached for 24 h in the presence of either DMSO, 100 μ M DFO, 100 μ M α -tocopherol, or 500 μ M Trolox, and the number of viable cells was quantified. (E) MCF10-A or SUM-159 vector control and $\beta 4$ -depleted cells were assayed for LDH after 6 h of detachment with either DMSO or 2 μ M ferrostatin-1. (F) Vector and $\beta 4$ -depleted cells were incubated for the times indicated with either DMSO or 2 μ M ferrostatin-1, and viable cells were quantified. (G) Vector and $\beta 4$ -depleted cells were detached for 24 h in the presence of either DMSO, 2 μ M ferrostatin-1, 25 μ M Z-VAD-FMK, or ferrostatin-1 and Z-VAD-FMK, and the number of viable cells was quantified. All experiments were performed independently three times and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

promoter and coding regions of MCF10A cells (Fig. 6 A). We also observed that STAT3 phosphorylation was decreased in $\alpha 6 \beta 4$ -depleted cells compared with control cells after erastin treatment (Fig. 6, B and C) and ECM detachment (Fig. 6, D and E). In addition, inhibition of STAT3 using 5,15-diphenylporphyrin (DPP) increased ACSL4 expression (Fig. 6 F). DPP had no effect on cell viability during the course of that assay (unpublished data). Collectively, these data indicate that $\alpha 6 \beta 4$ -mediated Src-STAT3 activation represses expression of ACSL4, rendering the cell unable to undergo ferroptosis.

ECM detachment requires an up-regulation of GPX4 to avoid ferroptosis

One question that arises from these data is why adherent, $\alpha 6 \beta 4$ -depleted cells require erastin treatment to induce ferroptosis, whereas the same cells in ECM-detached conditions undergo ferroptosis spontaneously? As mentioned, ACSL4 is necessary, but not sufficient, for ferroptosis (Doll et al., 2017). Therefore, we sought to identify a regulator of ferroptosis that was altered in detached, but not adherent, conditions. We focused on GPX4 because of its central role in ferroptosis and

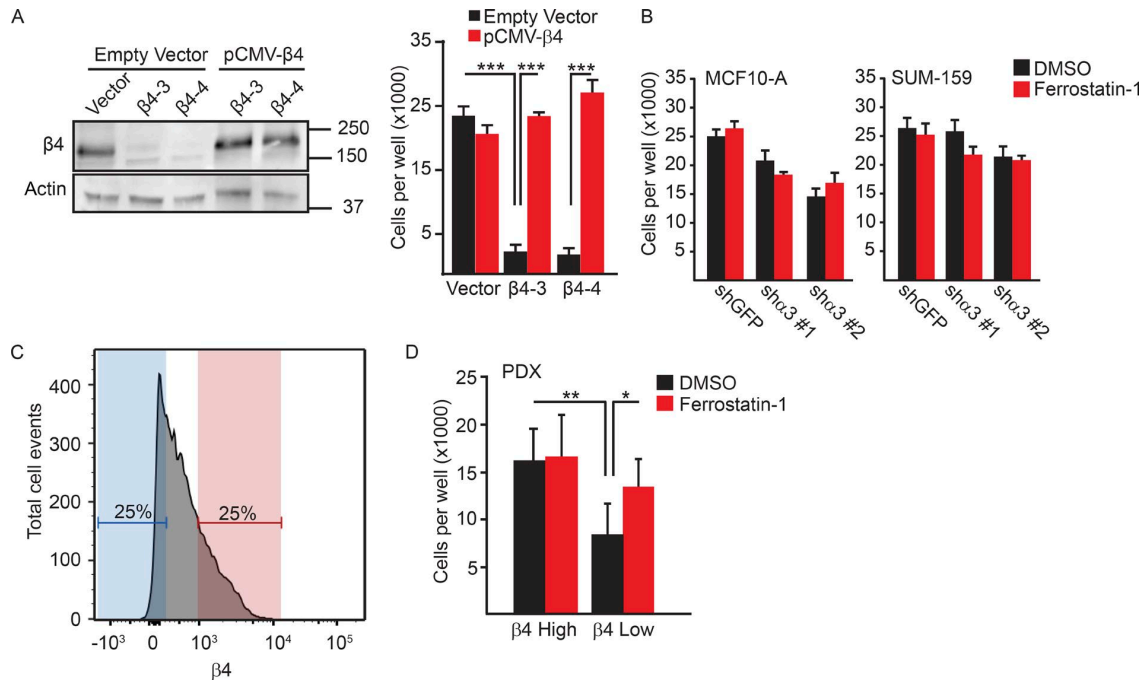


Figure 3. Evasion of ferroptosis is specific to the $\alpha 6 \beta 4$ -integrin. (A) $\beta 4$ -depleted SUM-159 cells were transfected with either a control plasmid (vector) or a $\beta 4$ expression construct in which the PAM sequences targeted by the guide RNAs were mutated. Rescue of $\beta 4$ expression was confirmed by immunoblotting and qPCR. Vector control cells, $\beta 4$ -depleted cells, vector control cells transfected with $\beta 4$ containing PAM mutations, and $\beta 4$ -depleted cells transfected with $\beta 4$ containing PAM mutations ($\beta 4$ -rescued cells) were detached for 24 h, and the number of viable cells was quantified. (B) MCF10-A and SUM-159 $\alpha 3$ -depleted cells were detached and compared with control cells for their viability after 24 h. (C and D) PDXs of triple-negative breast tumors were isolated, dissociated, and lineage-depleted, and the tumor cells were sorted based on the level of $\beta 4$ surface expression into $\beta 4^{\text{high}}$ (red) and $\beta 4^{\text{low}}$ (blue) populations. Those two populations were assessed for viability in detached conditions, either in the presence or absence of ferrostatin-1. All experiments were performed independently three times, and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

its ability to buffer lipid peroxidation. Moreover, GPX4 is the only glutathione peroxidase that accepts phospholipid hydroperoxides in membranes as oxidizing substrates (Thomas et al., 1990; Roveri et al., 1994; Seiler et al., 2008). There was no significant difference in GPX4 expression in adherent conditions between control and $\alpha 6 \beta 4$ -depleted cells (Fig. 7 A). However, we observed that ECM detachment caused an increase in GPX4 expression and that cells lacking $\alpha 6 \beta 4$ were unable to sustain GPX4 mRNA expression (Fig. 7 A). That inability to up-regulate GPX4 was also seen at the protein level, and expression levels were rescued with exogenous $\beta 4$ (Fig. 7 B).

Given that GPX4 buffers lipid peroxidation (Kriska and Girotti, 2005), we quantified lipid peroxidation in ECM-detached cells using the malondialdehyde (MDA) assay and observed that it was significantly greater in $\alpha 6 \beta 4$ -depleted cells than it was in control cells (Fig. 7 C). In adherent conditions, loss of $\alpha 6 \beta 4$ increases lipid peroxidation compared with vector control; however, the loss of ECM contact significantly increases the burden of lipid peroxidation in those $\alpha 6 \beta 4$ -depleted cells. Accordingly, glutathione peroxidase activity was elevated in detached, vector-controlled MCF10-A and SUM-159 cells compared with $\alpha 6 \beta 4$ -depleted cells (Fig. 7 D).

To test whether the loss of GPX4 was responsible for the ferroptosis observed in $\alpha 6 \beta 4$ -depleted cells upon ECM detachment, we expressed exogenous GPX4 in control and $\alpha 6 \beta 4$ -depleted cells (Fig. 7 E). Exogenous GPX4 expression reduced cell death significantly in ECM-detached MCF10-A and SUM-159 cells lacking $\alpha 6 \beta 4$, but it had no effect on the viability of control

cells (Fig. 7 F). Importantly, $\alpha 6 \beta 4$ -depleted cells that expressed exogenous GPX4 were sensitive to erastin, consistent with the fact that system X_c^- , which is the target of erastin, functions upstream of GPX4 (Yang and Stockwell, 2016; Fig. 7 G).

Discussion

This study advances our knowledge of ferroptosis with respect to physiologic processes that trigger this form of cell death and mechanisms used by cells to resist it. As discussed recently, ferroptosis occurs when a “cell is sabotaged by its own ongoing normal cellular metabolic activity, such as the production of lipid hydroperoxides” and that it can be prevented if these normal activities are inhibited (Dixon, 2017). Our data substantiate that assessment and reveal that physiological (ECM detachment), as well as chemical (erastin), stimuli induce ferroptosis in epithelial and carcinoma cells. We also discovered a novel mechanism used by those cells to prevent such metabolic disruption that involves the $\alpha 6 \beta 4$ integrin. The fact that $\alpha 6 \beta 4$ expression is characteristic of epithelial and carcinoma cells suggests that those cells have acquired mechanisms to mitigate metabolic stresses that can trigger ferroptosis. Moreover, the ability of $\alpha 6 \beta 4$ to evade ferroptosis is not shared by other integrins expressed by those cells, including $\alpha 6 \beta 1$ and $\alpha 3 \beta 1$, based on our findings.

The key mechanism that we uncovered for the evasion of ferroptosis by $\alpha 6 \beta 4$ involves its ability to activate Src in stress

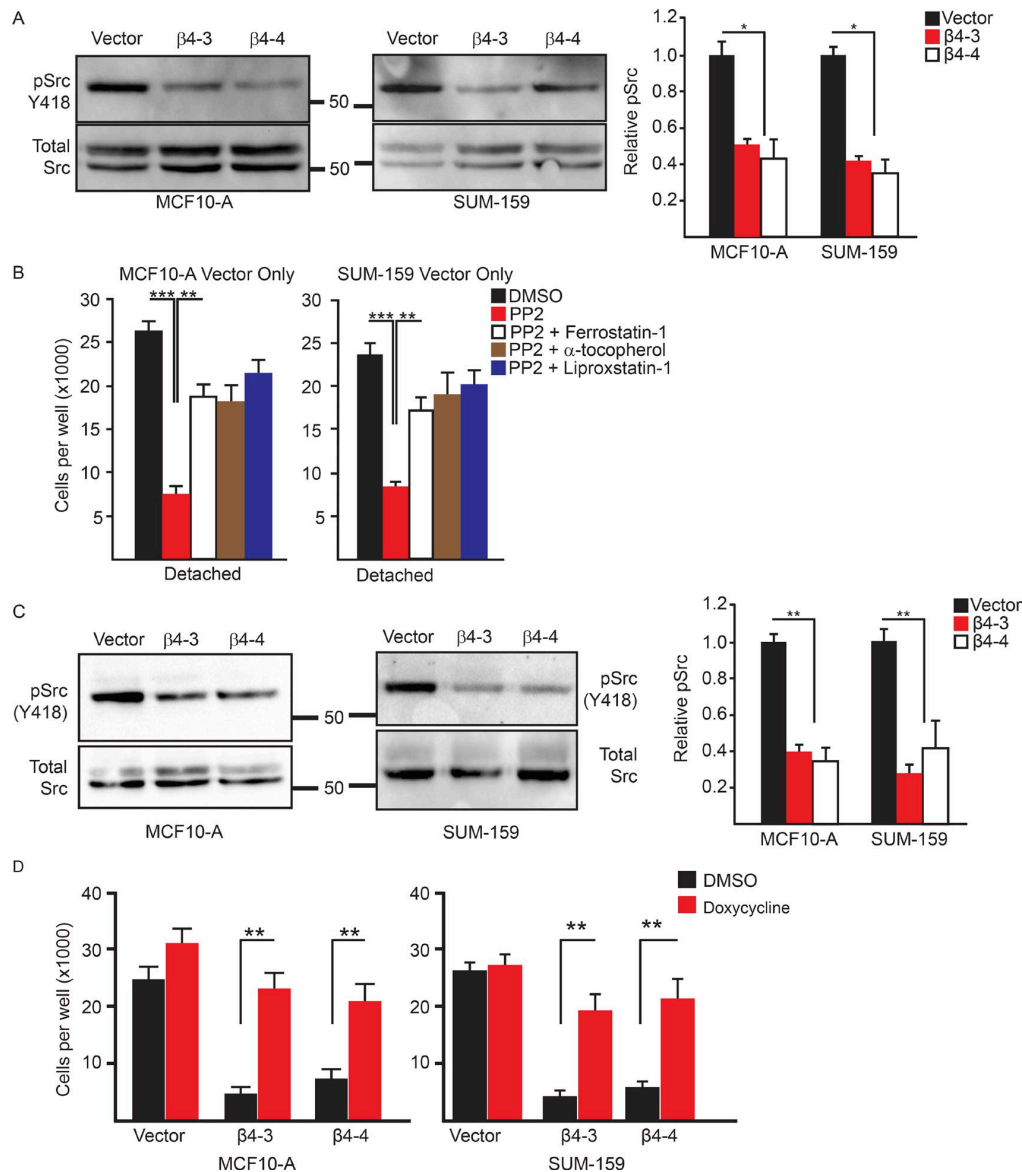


Figure 4. The integrin $\alpha 6\beta 4$ activates Src to inhibit ferroptosis. (A) Vector control and $\beta 4$ -depleted cells were assessed for phosphorylated (Y418) Src by immunoblotting 3 h after ECM detachment. Relative phosphorylated Src was quantified by densitometry. (B) Vector control and $\beta 4$ -depleted cells were assessed for viability after 24 h of detachment in the presence of either DMSO, 10 μ M PP2, PP2 and 2 μ M ferrostatin-1, PP2 and 500 μ M α -tocopherol, or PP2 and 2 μ M liproxstatin-1. (C) Vector control and $\beta 4$ -depleted cells were assessed for phosphorylated (Y418) Src by immunoblotting after 3 h of incubation with 10 μ M erastin. Relative phosphorylated Src was quantified by densitometry. (D) SUM-159 vector control and $\beta 4$ -depleted cells that expressed a doxycycline-inducible, constitutively active Src were incubated for 24 h with 2 μ g/ml doxycycline and then assessed for viability after 24 h of detachment. All experiments were performed independently three times, and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

conditions and the consequent Src-mediated repression of ACSL4. ACSL4 generates a proferroptotic lipid composition in the plasma membrane, which is characterized by increased expression of long polyunsaturated $\omega 6$ fatty acids (Doll et al., 2017), and its repression nullifies the ferroptotic response (Doll et al., 2017). The $\alpha 6\beta 4$ -Src signaling axis is well established, and it has been implicated in the diverse functions associated with this integrin (Gagnoux-Palacios et al., 2003; Bertotti et al., 2006; Merdek et al., 2007; Dutta and Shaw, 2008; Yang et al., 2010; Sharma et al., 2012; Pavlova et al., 2013; Hoshino et

al., 2015). The ability of Src to evade ferroptosis by repressing ACSL4 via the activation of STAT3, however, is novel and it provides one of the first examples of a major signaling mechanism that has the ability to resist the altered metabolic activity that can trigger this form of cell death. We demonstrate that $\alpha 6\beta 4$ -mediated Src activation contributes to ferroptosis resistance, but it is likely that other modes of Src activation result in such resistance, depending on the cellular context. Although not addressed directly, it is likely that $\alpha 6\beta 4$ -mediated Src activation in ECM-detached cells is independent of ligand

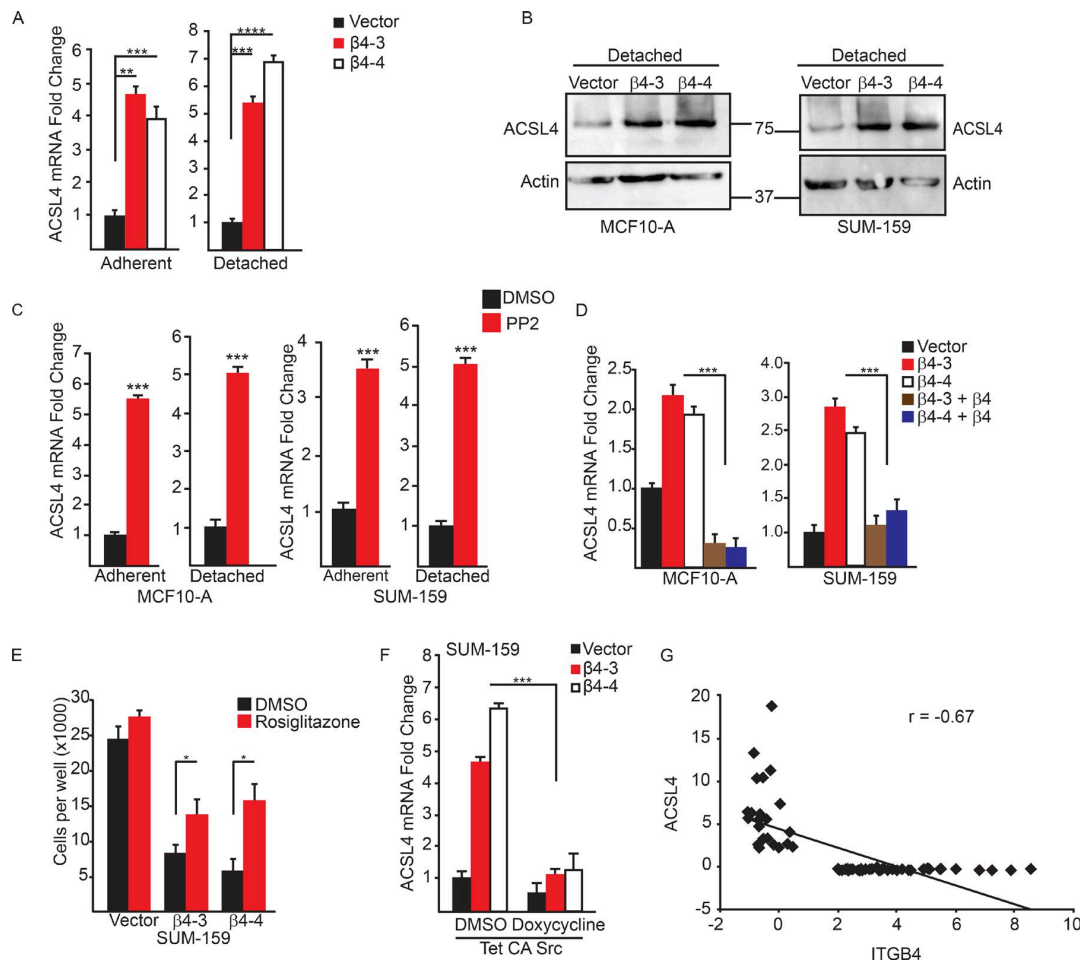


Figure 5. The integrin $\alpha 6 \beta 4$ represses ACSL4 to inhibit ferroptosis. Expression of ACSL4 was assessed by qPCR (A) and immunoblotting (B) in vector control and $\beta 4$ -depleted cells after 2 h of ECM detachment. (C) Expression of ACSL4 was quantified by qPCR in vector control MCF10-A or SUM-159 cells under either adherent or detached conditions after 2 h of treatment with DMSO or 10 μ M PP2. (D) Expression of ACSL4 was quantified in adherent vector control, $\beta 4$ -depleted, and $\beta 4$ -rescued cells by qPCR. (E) Vector control and $\beta 4$ -depleted cells were assessed for viability after 24 h of detachment in the presence of either DMSO or the ACSL4 inhibitor 5 μ M rosiglitazone. (F) SUM-159 vector control and $\beta 4$ -depleted adherent cells that expressed a doxycycline-inducible, constitutively active Src were incubated for 24 h with 2 μ g/ml doxycycline, and the expression of ACSL4 was quantified by qPCR. (G) Expression of ITGB4 and ACSL4 was correlated using a published gene expression database (cBioportal) comprising 70 human breast tumors. The correlation coefficient (r) was calculated using Pearson's correlation. Experiments were performed independently three times, and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

(laminin), based on previous studies that addressed this issue (Pavlova et al., 2013).

Our finding that ECM detachment triggers ferroptosis is significant because little is known about physiologic or pathophysiologic mechanisms that are linked to this cell death process. Moreover, it is widely assumed that cells undergo apoptosis in response to ECM detachment (Meredith et al., 1993; Frisch and Francis, 1994), a phenomenon referred to as anoikis (Frisch and Francis, 1994). Although the discovery of anoikis has been a significant contribution to the field, the relationships among ECM detachment and cell death mechanisms are more complex and anoikis-independent pathways are also involved (Buchheit et al., 2014). There is also evidence that ECM detachment can increase intracellular ROS and cause ROS-dependent cell death (Schafer et al., 2009), although the mechanisms involved are not well understood (Buchheit et al., 2014). Our discovery that ECM detachment can trigger ferro-

ptosis provides one mechanism for these results. As demonstrated here, ECM-detached cells are prone to both ferroptosis and apoptosis in the absence of $\alpha 6 \beta 4$. The apoptosis result is consistent with previous studies on this integrin (Bachelder et al., 1999; Zahir et al., 2003). Going forward, it will be important to determine the distinction between these two processes, particularly the point at which a decision is made by a cell to undergo either ferroptosis or apoptosis.

Although GPX4 is emerging as the critical gatekeeper of ferroptosis (Yang et al., 2014; Yang and Stockwell, 2016), biological mechanisms that regulate its expression or activity are poorly understood. In this direction, our findings substantiate the hypothesis that ferroptosis requires both GPX4 inhibition and ACSL4 activity. Adherent cells lacking $\alpha 6 \beta 4$ exhibit a significant increase in ACSL4 expression, but they are not prone to ferroptosis because GPX4 expression is sustained and it impedes lipid peroxidation. For that reason, these cells require an

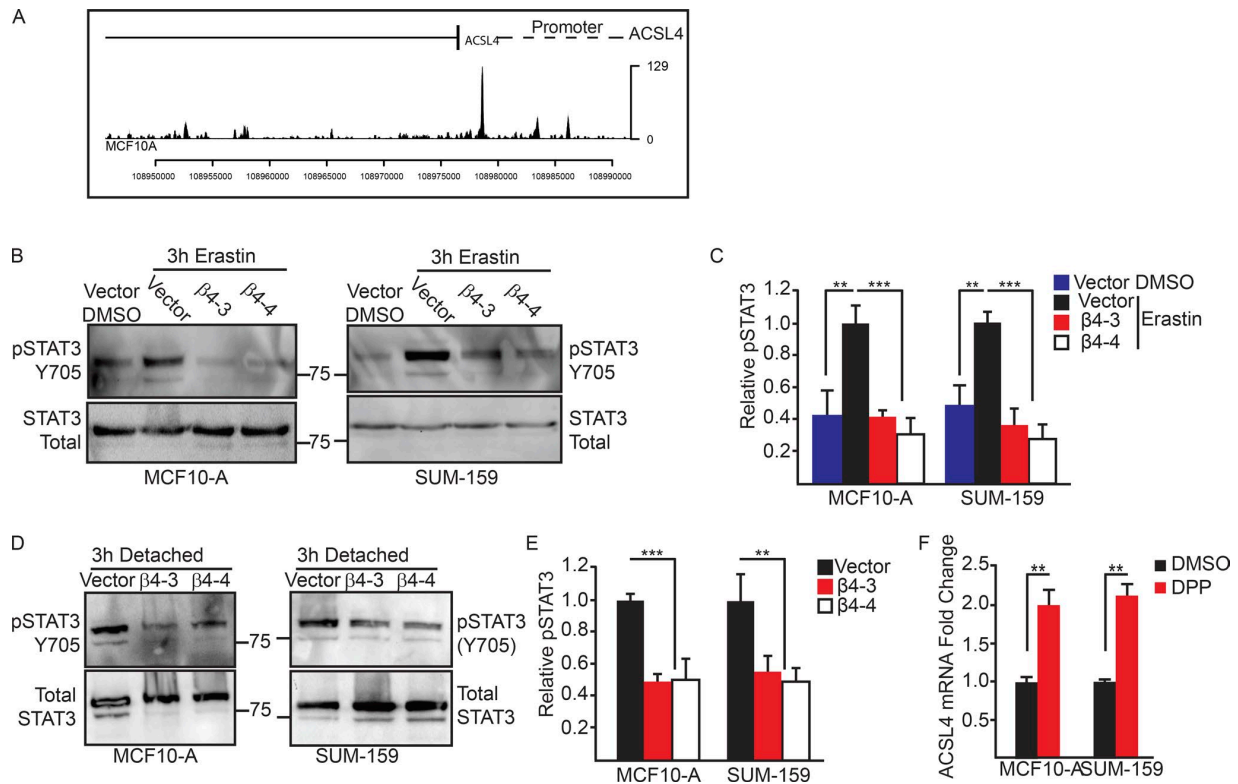


Figure 6. STAT3 represses transcription of ACSL4 downstream of the integrin $\alpha 6\beta 4$. (A) Analysis of MCF10-A cells using the ENCODE database showed binding of STAT3 to the promoter region. The phosphorylation of STAT3 was assessed by immunoblotting in vector control and $\beta 4$ -depleted MCF10-A cells after 3 h of detachment (B) or after incubation with erastin (D) and was also quantified by densitometry (C and E). (F) Expression of ACSL4 was quantified by qPCR in vector control MCF10-A and SUM-159 adherent cells after 2 h of incubation with the STAT3 inhibitor DPP. Experiments were performed independently three times, and a representative experiment is shown. The bars in graphs represent means \pm SD. **, P < 0.01; ***, P < 0.005.

exogenous inhibitor of GPX4, such as erastin, to undergo ferroptosis. In contrast, ECM detachment is sufficient to trigger ferroptosis in the absence of $\alpha 6\beta 4$ because lipid peroxidation increases as a result of ACSL4 induction and diminished GPX4 expression. Our data indicate that $\alpha 6\beta 4$ contributes to sustaining GPX4 expression in ECM-detached cells, but the mechanism appears to be distinct from $\alpha 6\beta 4$ -mediated repression of ACSL4 by Src because Src inhibition does not affect GPX4 expression (unpublished data).

The findings we present have potential implications for breast and other cancers. Although much is known about the mechanisms by which $\alpha 6\beta 4$ contributes to epithelial biology and breast cancer, a cohesive mechanism has not emerged, and the possibility that this integrin protects tumor cells from excessive lipid peroxidation has not been considered. This mechanism is likely to be important for metastasis because this process involves detachment from ECM or the presence of the “foreign” ECM in a distant organ (Cheung and Ewald, 2016). Moreover, we detected a significant inverse correlation between $\alpha 6\beta 4$ and ACSL4 in a large cohort of patients with breast cancer, and we demonstrated that the $\beta 4^{\text{low}}$ population isolated from PDXs of human breast cancer is much more susceptible to ferroptosis induced by ECM detachment than is the $\beta 4^{\text{high}}$ population. This latter observation is of particular interest because some chemotherapeutic drugs, as well as more novel therapies, such as the use of nanoparticles to deliver tumor-targeting peptides, function by inducing

ferroptosis (Yang et al., 2014; Kim et al., 2016). Our data suggest that tumor cells with high $\alpha 6\beta 4$ expression could be resistant to such therapies.

Materials and methods

Cell lines and reagents

MCF10-A cells were obtained from the Barbara Ann Karmanos Cancer Institute, and SUM-159 cells were provided by S. Ethier (Medical College of South Carolina, Charleston, SC). Hs578T cells were provided by D. Kim (University of Massachusetts Medical School, Worcester, MA). The pCMV- $\beta 4$ plasmid was provided by L. Shaw (University of Massachusetts Medical School, Worcester, MA). A Tet-CA-Src-GFP construct was purchased from Addgene (plasmid 83469). All cells were checked quarterly for mycoplasma.

The following antibodies were used: GPX4 (Abcam), actin (Sigma-Aldrich), integrin $\beta 4$ (505 [Rabinovitz et al., 1999] and 439-9b [Abcam]), $\alpha 6$ (GoH3; MilliporeSigma), and integrin $\beta 1$ (BD), phospho-Src Y418 (R&D Systems), total Src (Santa Cruz Biotechnology, Inc.), ACSL4 (Santa Cruz Biotechnology, Inc.), phospho-STAT3 Y705 (Cell Signaling Technology), and total STAT3 (Cell Signaling Technology). Other reagents used were: Z-VAD-FMK (SelleckChem), erastin (Sigma-Aldrich), ferrostatin-1 (Sigma-Aldrich), liproxstatin-1 (Sigma-Aldrich), DFO mesylate salt (Sigma-Aldrich), α -tocopherol (Sigma-Aldrich), trolox (Sigma-Aldrich), DPP (Sigma-Aldrich), and rosiglitazone (Tocris Bioscience).

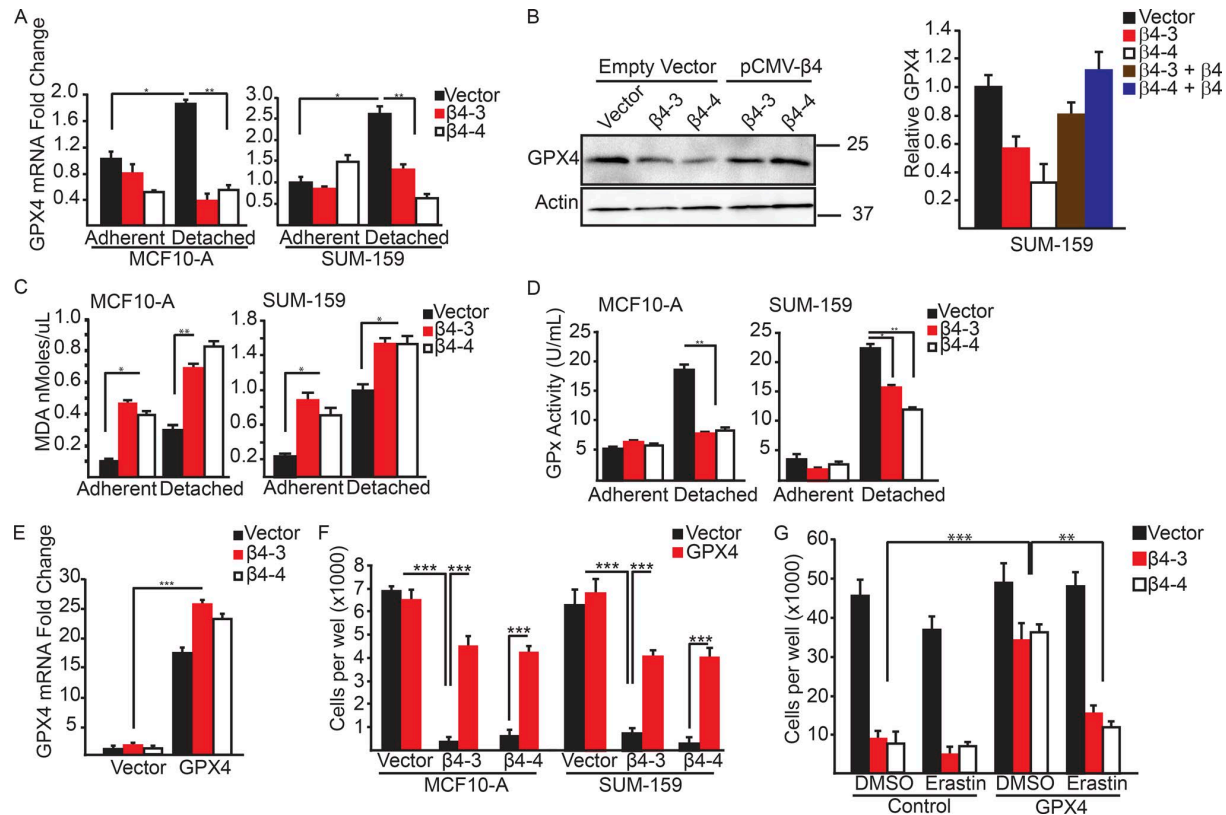


Figure 7. Matrix-deprived cells exhibit increased lipid peroxidation and an inability to sustain GPX4 expression in the absence of the $\alpha 6 \beta 4$ -integrin. (A) GPX4 mRNA expression was quantified by qPCR in control and $\beta 4$ -depleted MCF10-A and SUM-159 cells under adherent or 2-h matrix-deprived conditions. (B) Expression of GPX4 was assessed by immunoblotting in vector control, $\beta 4$ -depleted, and $\beta 4$ -rescued cells after 2 h of detachment. Relative densitometry values are shown. (C) Lipid peroxidation was quantified using the MDA assay in control and $\beta 4$ -depleted, MCF10-A and SUM-159 cells under adherent or 4-h matrix-deprived conditions. (D) GPX enzyme activity was assayed in control and $\beta 4$ -depleted, MCF10-A and SUM-159 cells under adherent or 4-h matrix-deprived conditions. (E) Control and $\beta 4$ -depleted SUM-159 cells were transfected with either a vector control or a GPX4 expression vector. GPX4 mRNA expression was quantified by qPCR. (F) Control and $\beta 4$ -depleted MCF10-A and SUM-159 cells that had been transfected with either a vector control or a GPX4 expression vector were detached for 24 h, and the number of viable cells was quantified. (G) Control and $\beta 4$ -depleted cells that had been transfected with either a vector control or a GPX4 expression vector were detached for 24 h with the presence of either DMSO or 10 μ M erastin, and the number of viable cells was quantified. Experiments were performed independently three times and a representative experiment is shown. The bars in graphs represent means \pm SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

RNA interference

Lentiviruses containing ITGA3 shRNAs (clone ID TRCN0000057713 or TRCN0000057714; GE Healthcare) or a GFP control (RHS4459) were generated, titrated according to the manufacturer's instructions, and used to transiently infect MCF10-A and SUM-159 vector control and $\beta 4$ -depleted cells, following standard protocols.

PDX tumors

PDX models of triple-negative breast cancer were obtained from the Dana-Farber Cancer Institute and propagated in NSG mice. Tumors were harvested and digested using collagenase at 37°C. Once digested, the cells were filtered using a cell strainer (40 μ m), washed twice with PBS, and plated in DMEM/F12 (containing 10% FBS). The next day, cells were stained using a $\beta 4$ antibody for 1 h and analyzed by flow cytometry. The highest and lowest quartiles of cells expressing $\beta 4$ were collected and plated immediately for experimentation.

Colony formation assay

MCF10-A and SUM-159 vector control or $\beta 4$ -depleted cells (5×10^2) were plated on 60-mm dishes. 24 h after plating, cells were incubated in medium containing erastin (10 μ M), erastin and ferrostatin-1 (2 μ M), or

DMSO control. Media were changed daily with continued exposure. 10 d after the first treatment, plates were washed in PBS, fixed in 4% paraformaldehyde, and stained in crystal violet. Plates were washed clean of excess dye and colonies >50 cells were counted. For some experiments, the crystal violet-stained cells were solubilized with DMSO, and absorbance was read at 590 nm.

Matrix-detachment assays

24-well plates were coated with poly 2-hydroxyethyl methacrylate (polyHEMA; 30 mg/ml; Sigma-Aldrich) and dried overnight. Trypsinized cells were plated (2.5×10^4 per well) in serum-free medium with the reagents indicated in the figure legends for the times noted. Cells were counted with a hemocytometer using trypan blue exclusion. Total cell number was calculated by multiplying the mean of cells per square by the dilution factor and chamber volume.

Biochemical experiments

For immunoblotting, cells were extracted using radioimmunoprecipitation assay buffer containing protease inhibitors (Boston BioProducts). Extracts were separated by SDS-PAGE and immunoblotted using the antibodies specified in the figure legends. Immunoprecipitation was

performed on precleared lysates by incubation with the primary antibody and, subsequently, protein G sepharose beads (Sigma-Aldrich). Immune complexes were dissociated, and proteins were separated by SDS-PAGE and immunoblotted as described in the figure legends. For qPCR, RNA was isolated from cells with NucleoSpin Gel and the PCR Clean-Up kit (Macherey-Nagel), and cDNAs were produced using the All-In-One cDNA Synthesis SuperMix (BioScript Solutions). qPCR was performed using a SYBR Green master mix (biotool.com). Sequences for primers used are provided in Table S1. The two-tailed Student *t* test was used to assess statistical significance.

To assess cytotoxicity, the LDH assay (Thermo Fisher Scientific) was used, according to the manufacturer's specifications. In brief, 5,000 cells were cultured in 96-well adherent or polyHEMA-coated plates in serum-free media for 4 h in DMSO, erastin (10 μ M), or ferrostatin-1 (2 μ M), as indicated. Medium from each well was reacted to form red formazan. Absorbance was read at 490 and 680 nm, and the LDH per well was calculated.

To assay lipid peroxidation, the MDA lipid peroxidation microplate assay (Sigma-Aldrich) was used according to manufacturer's specifications. In brief, cells (10^6) were cultured in serum-free medium on polyHEMA-coated plates for 4 h. Cells were collected, lysed, and reacted with thiobarbituric acid. Fluorescence was read at 532 (excitation) and 590 (emission). Lipid peroxidation levels were normalized to protein concentration. Glutathione peroxidase activity assay (BioVision) was performed according to the manufacturer's specifications. In brief, cells (10^6) were trypsinized and cultured in serum-free medium on polyHEMA-coated plates for 4 h. Cells were lysed and challenged with cumene hydroperoxide to assess the activity of glutathione peroxidase. Plates were read at 340 nm 5 min after challenge. Glutathione peroxidase activity was normalized to protein concentration.

Molecular biology experiments

For CRISPR-mediated deletion of the $\beta 4$ integrin subunit, guide RNAs targeting exon 1 of the $\beta 4$ sequence were selected using two websites, CRISPR Design (<http://crispr.mit.edu>) and CRISPRdirect (<https://crispr.dbcls.jp>). Four guide RNAs were tested, and the two most efficient knockouts were selected and used to control for potential off-target effects. Cells were subcloned by FACS and screened for loss of protein expression by immunoblot. To rescue the $\beta 4$ deletion, the protospacer-adjacent motif (PAM) sequences targeted by the guide RNAs were mutated using the New England Biolabs, Inc., Q5 site-directed mutagenesis kit in pcDNA3.1-Myc- $\beta 4$ (16039; Addgene). Mutagenesis primers were designed using the New England BioLabs, Inc., BaseChanger tool. Silent mutations were used to prevent changes in the amino acid sequence (Table S1). To express constitutively active Src, MCF10-A and SUM-159 vector control or $\beta 4$ -depleted cells were incubated with lentiviral-packaged Tet-CA-Src-GFP for 24 h before selection with 2 μ g/ml puromycin for 7 d. Src expression was induced by 24-h incubation with 2 μ g/ml doxycycline. To express GPX4, a plasmid construct for GPX4 was purchased from OriGene (RC208065). Cells were transfected with 10 μ g DNA using Lipofectamine 2000 (Thermo Fisher Scientific) and incubated for 48 h before use.

ENCODE database analysis

The signal of the STAT3 chromatin immunoprecipitation sequencing on the human MCF10A cell line (a stably expressed Er-Src fusion protein treated with 0.01% ethanol) was downloaded from the ENCODE project (<https://www.encodeproject.org/experiments/ENCSR000DOZ/>; National Human Genome Research Institute). The track was plotted with the Bioconductor trackViewer (version 1.12.0) package.

Statistical analysis

The bars in graphs represent means \pm SD. All experiments were repeated at least three times. The *p*-value was calculated using Student's *t* test and a *p*-value <0.05 was considered significant.

Online supplemental material

Table S1 lists the primers used for qPCR and mutagenesis analyses.

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The authors declare no competing financial interests.

Author contributions: C.W. Brown and A.M. Mercurio conceived the study, designed the experiments, oversaw the project, and wrote the manuscript. C.W. Brown executed and interpreted the experiments. J.J. Amante provided technical assistance and prepared the final figures. H.L. Goel provided insightful input on the study and critical analysis of the data and contributed to writing the manuscript.

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